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Metabolism of 17-hydroxyprogesterone by a *Bacillus* species

Shashi B. MAHATO* and Sukdeb BANERJEE
Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Jadavpur, Calcutta-700 032, India

Fermentation of 17-hydroxyprogesterone with a *Bacillus* species (IICB-301) in a modified nutrient medium under aerobic conditions yielded androst-4-ene-3,17-dione and 15α,17-dihydroxypregn-4-ene-3,20-dione in addition to a new pregnane analogue, 6β,17,20α-trihydroxypregn-4-ene-3-one. Each microbial metabolite was characterized by the application of various spectroscopic techniques. The availability of the new metabolite, 6β,17,20α-trihydroxypregn-4-ene-3-one, enabled complete elucidation of its ^{13}C-n.m.r. spectrum.

INTRODUCTION

Microbial transformations are used as a general means to prepare steroid derivatives that are difficult to synthesize by chemical methods. In our previous studies on the preparation of various physiologically important steroid derivatives by microbial transformation (Mahato & Banerjee, 1980; Mahato *et al.*, 1981; Mukherjee *et al.*, 1982; Mukherjee & Mahato, 1984; Mahato & Mukherjee, 1984; Mahato *et al.*, 1984) we incubated 17-hydroxyprogesterone with a strain of a *Bacillus* species. Metabolism of progesterone and testosterone by this strain of *Bacillus* had been studied, and the isolation of a number of interesting metabolites was reported (Mahato *et al.*, 1984). It is noteworthy that no side-chain-cleavage product was obtained in the fermentation of progesterone by this strain. Fermentation of 17-hydroxyprogesterone with this strain, however, resulted in the formation of androst-4-ene-3,17-dione, a side-chain-cleavage product and two hydroxylated products, including a 20-oxo reduction compound which is a hitherto-unreported pregnane analogue. There are some fungal species, mainly of the genera *Fusarium*, *Aspergillus*, *Streptomyces*, *Penicillium*, *Mucor*, *Gliocladium* and *Cylindrocarpon*, that are capable of stereospecifically degrading the C-17β acetyl side chain of progesterone and related analogues. However, different micro-organisms appear to have altered specificity with regard to the C-17 side chain. Whereas some organisms are unable to attack the steroid side chain bearing substituents at C-16 or C-17, some other organisms are unaffected by substituents at these positions. Although hydroxylation reactions by various *Bacillus* species are of common occurrence (Smith, 1974), no side-chain cleavage by any *Bacillus* species has yet been reported. Moreover, this is the first report of microbial transformation of a steroid involving hydroxylation, reduction of oxo group and cleavage of the C-17β acetyl side chain.

MATERIALS AND METHODS

Materials

Inorganic salts were purchased from Sarabhai Chemicals Ltd., Bombay, India. Peptone, beef extract, yeast extract and agar were purchased from Difco Laboratories, Detroit, MI, U.S.A. Silica gel for column chromatography and for t.l.c. (60 HF_{254}; Merck) and organic solvents, were supplied by E. Merck, Bombay, India. 17-Hydroxyprogesterone was the product of Sigma Chemical Co., St. Louis, MO, U.S.A. Reagents and solvents were of analytical grade. 17-Hydroxyprogesterone was re-crystallized before use.

Fermentation methods

The strain of *Bacillus* species (Registry no. IICB-301) was being maintained in nutrient-agar slants at the Culture Collection Unit of the Steroid and Terpenoid Division of this Institute. Fermentation of 17-hydroxyprogesterone was carried out in Erlenmeyer flasks (2 litres), each containing 400 ml of the following medium: peptone, 5 g; yeast extract, 2.5 g; beef extract, 2.5 g; NaCl, 2.5 g; distilled water, 1 litre (pH adjusted with NaOH to 7.0). A total of 1 g of 17-hydroxyprogesterone dissolved in acetone (70 ml) was evenly distributed among five flasks (2 litres) containing the medium, plugged with cottonwool and sterilized by autoclaving at 121 °C for 15 min. The acetone was lost by evaporation during 15 min at 121 °C. Inoculation of the sterilized medium containing the substrate was made with a cell suspension obtained from 18 h-old cultures maintained on nutrient-agar slants. The batch of five flasks thus inoculated was incubated on a rotary shaker at 100 rev./min at 37 °C for 96 h.

Extraction and isolation of the metabolites

After 96 h of incubation the fermentation mixture was harvested and exhaustively extracted thrice with equal volumes of chloroform. The organic layer was washed with distilled water and dried over anhydrous Na_2SO_4. After complete evaporation of solvent, a brownish semi-solid residue (0.993 g) was obtained. T.l.c. examination of the residue on silica-gel plates (Merck 60 HF_{254}), with chloroform/ethyl acetate/methanol (20:4:1, by vol.) as the developing solvent system, revealed the formation of three metabolites. The residue was chromatographed on a column of silica gel (25 g). The elution was successively with light petroleum (b.p. 60–80°C)/ethyl acetate (3:1, v/v), light petroleum/ethyl acetate (1:1, v/v) and ethyl acetate/methanol (19:1, v/v). The fractions obtained were further purified by rechromatography and preparative t.l.c., followed by crystallization. Thus unconverted 17-hydroxyprogester-

* To whom correspondence and reprint requests should be addressed.

one (448 mg), metabolite A (compound **II**, Scheme 1 below) (79 mg), metabolite B (**III**) (117 mg) and metabolite C (**IV**) (246 mg) were isolated. T.l.c. examination of the ethyl acetate extract of the culture did not indicate the formation of any other polar metabolite. Thus about 89% of the 17-hydroxyprogesterone was accounted for and the rest was lost. No transformed product was obtained from the control.

Analytical methods

Melting points were obtained in open capillary tubes in an H_2SO_4 bath and are uncorrected. Optical rotations were measured in chloroform on a Perkin–Elmer automatic polarimeter. I.r. spectra were recorded on a Perkin–Elmer 177 spectrophotometer in Nujol nulls. Mass spectra were obtained with a Hitachi model RMU-6L mass spectrometer at an ionizing potential of 70 eV. ^{1}H- and ^{13}C-n.m.r. spectra were obtained with a JEOL-FT-100 spectrometer at 100 and 25.05 MHz respectively, with tetramethylsilane as an internal standard.

RESULTS AND DISCUSSION

Incubation of 17-hydroxyprogesterone with the strain of *Bacillus* species was carried out for 24, 48, 72 and 96 h. It was observed that better yields of the metabolites were obtained after 96 h of incubation. The isolated metabolites A (**II**, Scheme 1 below), B (**III**) and C (**IV**) were characterized from their physical and spectral data.

Metabolite A (**II**) was identified as androst-4-ene-3,17-dione by its i.r., m.s. and ^{1}H n.m.r. and also by comparison of its m.p. and specific rotation with an authentic sample (Mahato *et al.*, 1984).

Metabolite B (**III**), $C_{21}H_{30}O_4$, m.p. 194–196 °C, $[\alpha]_D$ +60° (*c* 0.52 in chloroform), exhibited in its i.r. spectrum characteristic bands at 3600 and 3455 (hydroxy), 1705 (saturated ketone), 1660 and 1612 cm^{-1} (α,β-unsaturated ketone). The mass spectrum showed the molecular-ion peak at *m/z* 346 and the significant fragment ion peaks at *m/z* (relative intensity) 331(17), 329(2), 313(9), 299(41), 287(100), 285(78), 269(39), 267(14) and 229(57). The data clearly indicated the introduction of a hydroxy group in (**I**). The ^{1}H n.m.r. spectrum, which suggested the position and orientation of the introduced hydroxy group (Bhacca & Williams, 1964), displayed chemical-shift signals at 0.76 (3 H, s, *H*-18), 1.18 (3 H, s, *H*-19), 2.17 (3 H, s, *H*-21), 3.84 (1 H, q, *H*-15) and 5.74 (1 H, s, *H*-4) p.p.m. Compound (**III**) was characterized as 15α,17-dihydroxypregn-4-ene-3,20-dione by comparison of its m.p., i.r. and ^{1}H n.m.r. with those reported in the literature (Tschesche *et al.*, 1967) for a chemically synthesized compound. It may be mentioned that this is the first report of its microbial preparation.

Metabolite C (**IV**) crystallized from ethyl acetate as prisms, m.p. 212–214 °C, $[\alpha]_D$ −13.5° (*c* 0.45 in chloroform). Elemental analysis and M_r determination by m.s. revealed the molecular formula to be $C_{21}H_{32}O_4$. The i.r. spectrum showed a strong absorption band at 1660 cm^{-1} attributable to the Δ^4-3-ketone, but the spectrum was transparent in the saturated-carbonyl-group region. The ^{1}H-n.m.r. spectrum also did not show the singlet corresponding to a 21-methyl group. It was apparent, therefore, that the C-20 ketone had been reduced. The function of the introduced oxygen atom was determined to be hydroxy by its m.s., ^{1}H and ^{13}C n.m.r. properties. The ^{1}H-n.m.r. spectrum exhibited chemical-shift signals at 0.82 (3 H, s, *H*-18), 1.20 (3 H, d, J = 6 Hz, *H*-21), 1.38 (3 H, s, *H*-19), 3.87 (1 H, m, *H*-20), 4.38 (1 h, t-like, *H*-6) and 5.84 (1 H, s, *H*-4) p.p.m. The orientations of 6- and 20-hydroxy groups turned out to be β and α respectively, taking into consideration their effects on C-19 and C-18 methyl groups. The C-19 methyl group and H-4 of (**IV**) shifted downfield by 0.2 and 0.1 p.p.m. respectively in comparison with that of (**I**), results which are in conformity with the presence of a C-6β OH group (Bhacca & Williams, 1964). The 18-methyl group of (**IV**) shifted downfield by 0.06 p.p.m. in comparison with that of (**I**), which is indicative of the C-20α OH group, because the C-18 methyl resonance frequencies of C-20 epimers are subjected to greater deshielding by the C-20 oxygen atom, and the effect is about +0.15 p.p.m. (Lee & Wolff, 1967). The m.s. of (**IV**) displayed peaks at *m/z* (relative intensity) 348(M^+, 3), 330 ($M^+ - H_2O$, 7), 312 ($M^+ - 2H_2O$, 3), 303 (M^+ − side chain, 25), 285 (M^+ − side chain − H_2O, 50), 267 (M^+ − side chain − $2H_2O$, 15), 245 (6), 242 (8), 239 (M^+ − side chain − $2H_2O$ − CO, 7), 227 (10), 225 (9), 223 (16), 205 (11) and 99 (100). The ^{13}C n.m.r. chemical shifts were consistent with the structure of the metabolite C (**IV**) shown. All the carbon resonances of (**IV**) were assigned by comparison with the ^{13}C data of (**I**) (Blunt & Stothers, 1977). The doublet at 71.0 was assigned to C-6, which shows a downfield shift of 39.1 p.p.m. (α-effect). The quaternary carbon C-5 and methylene carbon C-7 were shifted by −2.3 and +8.4 p.p.m. respectively (β-effect). The carbons C-8 and C-4 were shifted by −7.1 and +1.8 p.p.m. (α-effect). The doublet

Table 1. ^{13}C chemical shifts of 6β,17,20α-trihydroxypregn-4-ene-3-one (IV)

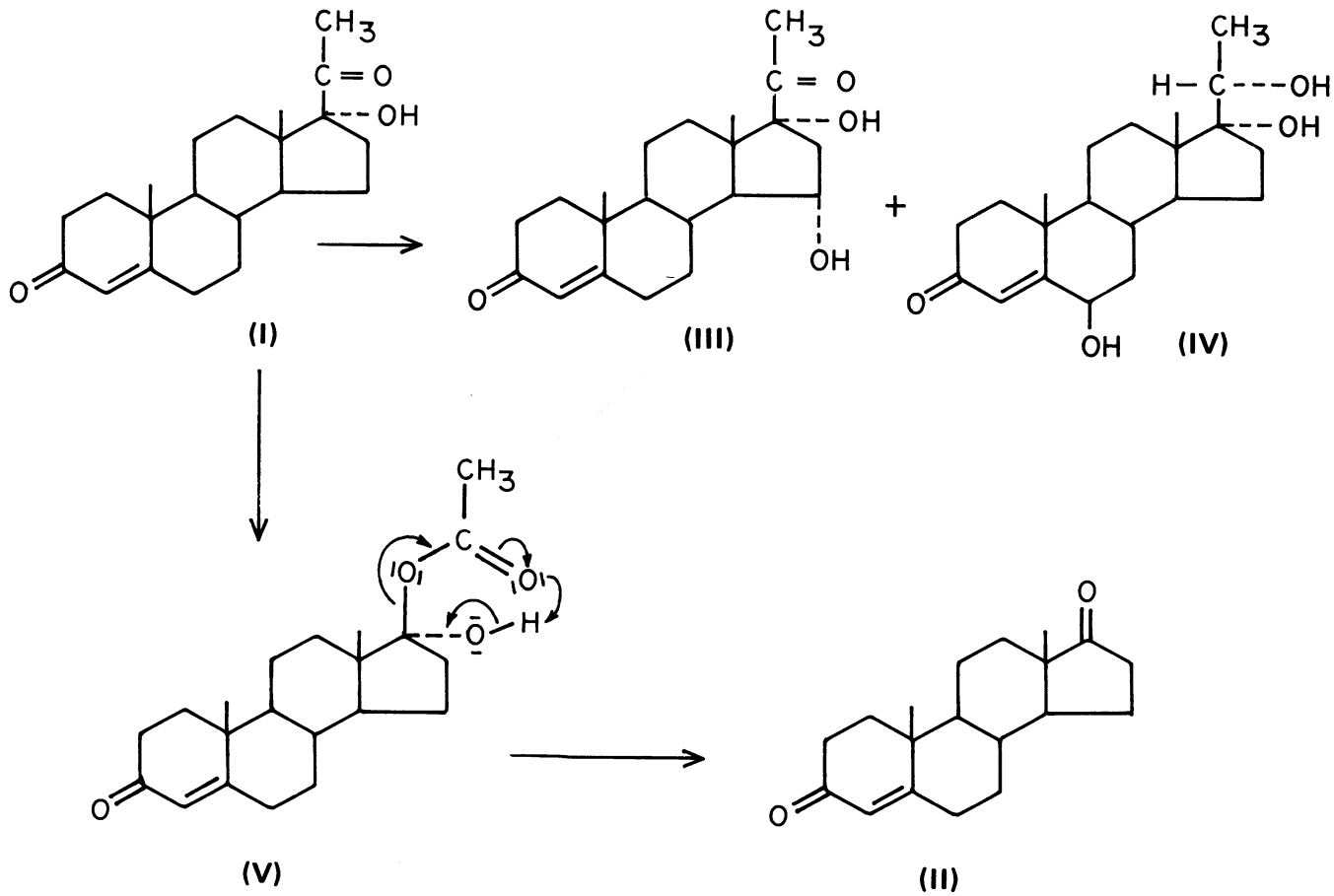
The spectrum was obtained at 25.05 MHz in [$^2H_6$]dimethyl sulphoxide and the values are given in p.p.m. with respect to the tetramethylsilane used as an internal standard. Values marked [b] and [c] may be interchanged.

Carbon atom no.	Chemical shifts (p.p.m.)		
	(I)*	(IV)	Δ(IV − I)
1	35.1	36.6[b]	(+1.5)
2	33.5	33.7	(+0.2)
3	198.2	198.8	(+0.6)
4	123.1	124.9	(+1.8)
5	171.2	168.9	(−2.3)
6	31.9	71.0	(+39.1)
7	32.0	40.4	(+8.4)
8	36.1	29.0	(−7.1)
9	53.1	52.9	(−0.2)
10	38.2	37.4	(−0.8)
11	20.3	19.9	(−0.4)
12	30.4	30.5	(+0.1)
13	46.3	45.0	(−1.3)
14	49.9	49.8	(−0.1)
15	23.1	22.89	(−0.3)
16	32.3	36.4[b]	(+4.1)
17	89.2	84.2	(−5.0)
18	14.5	13.9	(−0.6)
19	17.0	18.7[c]	(+1.7)
20	210.3	70.1	(−140.2)
21	26.6	18.5[c]	(−8.1)

* Blunt & Stothers (1977).

at 70.1 p.p.m. was assigned to C-20, which shows an upfield shift of −140.2 p.p.m. The C-21 carbon shifted upfield by 8.1 p.p.m. and resonated at 18.5 p.p.m. All the other carbons of (IV) were assigned (Table 1) taking into consideration the known chemical-shift rules (Wehrli & Wirthlin, 1978) and substituent effects (Eggert *et al.*, 1976). Treatment of (IV) with periodic acid followed by the usual workup resulted in the formation of a side-chain-cleavage product, m.p. 191–192 °C, which was characterized as 6β-hydroxyandrost-4-ene-3,17-dione (Holland & Diakow, 1978) by comparison with an authentic sample (m.p., ^{1}H n.m.r.). Thus the metabolite was characterized as 6β,17,20α-trihydroxypregn-4-ene-3-one (IV), which is a new pregnane analogue.

The formation of the three metabolites of 17-hydroxyprogesterone (I) by the enzyme system generated by the strain of the *Bacillus* species is of considerable interest. Whereas metabolite (III) is evidently formed by direct oxygenation at C-15, as is usually the case with microbial hydroxylation process, metabolite (IV) is produced by oxygenation at C-6 and reduction of the 20-oxo group. Moreover, metabolite (II) is formed by the degradation of the C-17β acetyl side chain of (I). In the microbial degradation of the progesterone side chain, molecular oxygen is directly inserted into the C-17–C-20 bond to give testosterone acetate (Nakano *et al.*, 1968), which is hydrolysed to testosterone and subsequently oxidized to (II). The pathway of formation of the side-chain-cleavage metabolites has been rationalized in the light of the non-enzymic Baeyer–Villiger type of oxidation of ketones to esters or lactones by peracids (Rahim & Sih, 1966; Miller, 1972; Carlstrom, 1973). The structure and mechanism of formation of the metabolites of (I) are shown in Scheme 1. The pathway of formation of the metabolite (II) may be visualized to have proceeded as follows: 17-hydroxyprogesterone (I) on enzymic Baeyer–Villiger type oxygenation yields the ester (V), which on non-enzymic rearrangement gives (II). The presence of the 17-OH group seemed to be essential for the production of (II), since no side-chain-cleavage metabolite was obtained when progesterone was used as substrate. On the other hand the presence of a hydroxy function at C-21 such as in cortexolone appeared to stimulate the rate of formation of (II). Moreover, the presence of either a 15α- or a 20α-hydroxy function completely inhibited the activity, as evidenced by the observation that the compounds (III) and (IV) were not metabolized when incubated with the strain under similar conditions. Although there are reports (Brannon *et al.*, 1965; Tan & Smith, 1968) of the formation of hydroxylated metabolites in addition to the side-chain-cleavage product, generation of such a combination of metabolites by any *Bacillus* species has not yet been reported. Moreover, formation of hydroxylated product along with a side-chain-cleavage metabolite and a 20-oxo reduction compound is a phenomenon which has not yet been reported in the literature. The generation of enzymes of such diverse characters by the same organism under similar conditions is of much interest and will help in understanding various biochemical phenomena.



Scheme 1. Structure and mechanism of formation of the metabolites of 17-hydroxyprogesterone by the *Bacillus* species

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